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Both IKK- α and IKK- β are implicated in the arsenite-induced AP-1 transactivation correlating with cell apoptosis through NF- κ B activity-independent manner

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Abstract

Arsenite has been well-proved to act as both an environmental carcinogen as well as a tumor therapeutic agent. AP-1 is one of the transcription factors that can be induced upon arsenite stimulation. However, the study on the mechanism and the function of the arsenite-induced AP-1 transactivation remains far complete. Here we demonstrated that high dose of arsenite-induced apoptotic response in mouse fibroblasts correlating with AP-1 transactivation, which events were mediated by both IKK α and IKK β , two major protein kinases responsible for NF- κ B activation. In addition, the regulatory effect of IKKs on the arsenite-induced AP-1 activation was delivered by sequential induction of GADD45 α expression and the activation of MAPKK (MKK3/4/6) and MAPK (JNKs and p38K)-dependent pathways. We further provided evidence that p50, but not of p65 subunit of NF- κ B, was involved in GADD45 α induction and the subsequent MAPKK/MAPK/AP-1 activation under arsenite exposure, while functional NF- κ B induced by arsenite stimulation was consist of p65 but not of p50 subunit. Therefore, we concluded that both IKK α and IKK β can mediate arsenite-induced AP-1 transactivation through NF- κ B activity-independent manner.

Keywords

IKK; AP-1; NF- κ B; cell death and arsenite

AP-1 (activation protein-1) transcription factor is a mixture of the homodimers or heterodimers composed of the basic region-leucine zipper (bZIP) proteins that mainly belong to the Jun (c-Jun, JunB, JunD), Fos (c-Fos, FosB, Fra-1, Fra-2) and ATF (ATF2, ATF3, B-ATF) protein subfamilies. Each of the proteins is differently expressed and regulated, so that the diverse combination of the AP-1 components gives rise to the multiple AP-1 dimeric pairs with different regulatory function [1–3].

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AP-1 activity can be induced by a plethora of physiological and pathological stimuli and is implicated in the different, and even opposing cellular process, such as tumor promotion and prevention, cell survival and apoptosis. The exact outcome of the AP-1 activation is highly tissue- and developmental stage-specific, which depends on the content of the downstream target genes activated by AP-1 [1–6]. The regulation of AP-1 activity occurs at the transcriptional and post-translational levels. In addition to the transcriptional induction of the AP-1 proteins, activation of AP-1 is also relied on a process of the inducible kinase-mediated phosphorylation of its components. The mitogen activated protein kinases (MAPKs), which include three subgroups of the ERKs, JNKs, and p38 MAPK, plays critical roles in this process [7]. The ERKs are usually involved in the activation of AP-1 induced by the mitogens and growth factors, and is important in cellular growth and differentiation [8,9]; while AP-1 activation by the pro-inflammatory cytokines and genotoxic stress is mainly mediated by the JNKs and p38 MAPK cascades and plays roles in regulating cell survival and apoptosis [7, 10,11].

NF- κ B is another kind of the transcriptional factor that also plays master role in modulating cell proliferation, differentiation, survival and apoptosis. The NF- κ B family are homo- or heterodimers that are formed from five structure-related protein subunits, p50 (NF- κ B1), p52 (NF- κ B2), p65 (RelA), c-Rel (Rel) and RelB. Usually, the NF- κ B dimers containing RelA, RelB or c-Rel subunit serve as the activators of the transcription due to the transcriptional activation domain (TAD) toward the C-terminal of these subunits; while the p50 or p52 homo- or hetero-dimers only can function as the transcriptional suppressor for the lack of the TAD domain. NF- κ B activity is normally tightly controlled by its inhibitor I- κ Bs, which binds to and retains NF- κ B in the cytoplasm in the resting cells. Stress stimulations trigger the activation of the specific I κ B kinases (IKKs) that contain two catalytic subunits named IKK α and IKK β and a regulatory subunit, IKK γ , and mediate I κ Bs phosphorylation and subsequently degradation, thereby releasing NF- κ B enter into the nucleus to regulate the transcription of a set of its downstream target genes [12,13]. The cross-talk between NF- κ B and AP-1 transcription pathways has been reported previously, under which conditions NF- κ B activation can positively or negatively modulate the secondary AP-1 transactivation through regulating the expression of the AP-1 components [14,15].

Arsenite is a well-documented carcinogen as well as a valuable tumor therapeutic agent [16, 17]. The previous published data from our group and others indicated that the dual role of arsenite in both tumor promotion and prevention seems mainly related to the exposure concentrations [18–21]. Low dose of arsenite induced cell proliferation accompanying with CyclinD1 upregulation [18,20]; while high level of arsenite triggered cell apoptosis by induction of GADD45 α expression [21]. AP-1 activity can be induced by either low or high dose of arsenite exposure in different tissues, indicated that AP-1 might act as a double-edged sword in the arsenite response [16,17]. However, the mechanism of arsenite-induced AP-1 activation remains elusive.

In this study, we focused on elucidating the molecular events that involved in the high dose of arsenite-induced AP-1 transactivation. We provided evidence that both IKK α and IKK β were mediators of high dose of arsenite-induced cell apoptotic effect and AP-1 transactivation in the mouse fibroblast. GADD45 α was the downstream target of IKKs to functionally link the MKK/JNKs-p38K pathways for the mediation of AP-1 induction. Furthermore, p50, but not of p65 subunit of NF- κ B, is critical for GADD45 α upregulation and the subsequent AP-1 transactivation; while the transcriptional activity of NF- κ B depends on p65 instead of the p50 subunit in response to the arsenite exposure, indicated that arsenite-induced AP-1 activation is dependent on IKKs but unrelated to the NF- κ B transcriptional activity.

Materials and methods

Cells and plasmids

IKK $\beta^{-/-}$ and IKK $\alpha^{-/-}$ MEFs and their corresponding wild type (WT) MEFs were provided by Dr. Michael Karin (University of California, San Diego, CA) [22]. The p50 $^{+/+}$, p50 $^{-/-}$, p65 $^{+/+}$, p65 $^{-/-}$ MEFs and the plasmid expressing p50 were provided by Dr. Jianping Ye (Louisiana State University, Baton Rouge, LA). The wild type and the gene knockout MEFs were maintained in DMEM (Calbiochem, San Diego, CA) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 2 mM L-glutamine (Life Technologies, Inc., Rockville, MD) at 37°C. The plasmids expressing full length IKK β (HA-IKK β), IKK α (FLAG-IKK α), dominant negative MKK4 (HA-DN-MKK4) and dominant negative MKK7 (HA-DN-MKK7) were provided by Dr. Zhenggang Liu (National Institutes of Health, Bethesda, ML) and described in the previous study [22]. The plasmid containing DN-c-Jun cDNA (TAM67) was described previously [19]. The AP-1-luciferase reporter plasmid was described previously [23].

Cell transfection

All of the stable and transient transfections were performed with LipofacTAMINE reagents (Life Technologies, Inc. Rockville, MD) according to the manufacturer's instructions. For stable transfection, cultures were subjected to either hygromycin B or G418 drugs selection and cells surviving from the selection were pooled as stable mass. These stable transfectants were cultured in the selective drug-free medium for at least two passages before subjecting to the according experiments.

Western Blot

Whole cell extracts were prepared with the cell lysis buffer (10 mM Tris-HCl, pH 7.4, 1% SDS, 1 mM Na₃VO₄). Protein concentrations were determined by the Bio-Rad protein quantification assay kit. Proteins (30 μ g) were resolved by SDS-PAGE, probed with the indicated primary antibodies, and then incubated with the AP-conjugated second antibody. Signals were detected by the ECF western blotting system as described in our previous report [19,21]. The images were acquired by scanning with the phosphorimager (model StormTM 860; Molecular Dynamics).

Luciferase reporter assay

MEFs with transient or stable transfection of the luciferase reporter constructs were seeded into 96-well plates (8 $\times$ 10³/well) and subjected to the various treatments when cultures reached 80–90% confluence. Cellular lysates were prepared at the indicated time-points and the luciferase activities were determined by a luminometer (Wallac 1420 Victor 2 multilable counter system) as described in our previous studies [19,23]. The results are expressed as relative activity which normalized to the control cells without treatment.

Electrophoretic Mobility Shift Assays (EMSA)

Nuclear proteins were prepared with the CellyticTM NuCLEARTM Extraction Kit (Sigma, St. Louis, MO) following the manufacturer's protocols and the gel shift assay was performed as described in our previous study [19,23].

The following synthetic oligonucleotides (5' to 3') containing the consensus or mutant NF- κ B binding sequences were used as probes/competitors in the EMSA experiments. NF- κ B consensus: GAGTTGAGGGGACTTTCCCAGGC. NF- κ B mutant: GAGTTGAGGTTACGGTCC CAGGC.

RESULTS

Both IKK α and IKK β are involved in the arsenite-induced AP-1 transactivation in MEFs

Arsenite can exert either cell proliferative or apoptotic effects in different cell types under various concentration of exposure [16,17]. Our recent report has demonstrated that high dose of arsenite treatment (20 μ M) can induce an apoptotic response in mouse fibroblasts, which effect was delivered by IKK β and NF- κ B p50 subunit-dependent manner [21]. AP-1 has been proved to be another key transcription factor that mediates arsenite-induced response in various target cells. And the final outcome of AP-1 transactivation under arsenite stimulation appears to be dose- and cell-type dependent, such as cell proliferation or cell apoptosis [16,17]. Therefore the aim of this study is to further address the role of AP-1 transactivation in the arsenite-induced cell apoptotic effect in MEFs and the relationship between AP-1 and IKK/ NF- κ B pathways activation in this process.

To this end, AP-1 luciferase reporter construct, in which the transcription of the luciferase reporter gene is driven by the inducible AP-1-responsive DNA elements, was firstly transfected into the wild-type, IKK α ^{-/-} and IKK β ^{-/-} MEFs (Fig. 1A) and the individual stable transfectants were established. When the cells were treated with 20 μ M of arsenite for the different time periods, at which dosage a pronounced cell death effect was induced according to our previous study [21], we observed an induction of the AP-1-dependent luciferase activity in WT cells as early as 6h and peaked at 12h post arsenite stimulation; whereas, either IKK α or IKK β deficiency rendered the dramatically declined AP-1 transactivation at all indicted time points under the same conditions (Fig. 1B), suggesting the involvement of both IKKs in the arsenite-induced AP-1 transactivation. Under the same conditions, arsenite-induced cell death was almost totally blocked in the IKK α ^{-/-} and IKK β ^{-/-} MEFs (Fig. 1C and 1D). Taken together, these results indicated that both IKK α and IKK β are the mediators of high dose of arsenite-induced AP-1 transactivation and the accompanying cell apoptotic response.

It has been well-identified that AP-1 dimers are consisted of Jun, Fos or ATF family proteins [1–3]. We next determined which AP-1 proteins were involved in the IKKs-mediated arsenite response mentioned above. As shown in Fig. 1E, phosphorylation of c-Jun and ATF2 and the upregulation of Fra-1 can be observed in the wild-type MEFs upon 20 μ M of arsenite exposure. There were no obvious alteration of other AP-1 components (including JunB, JunD, c-Fos, FosB and Fra-2) in the wild-type cells under the same conditions. Furthermore, the events of the inducible phosphorylations of c-Jun, ATF2 and the upregulation of Fra-1 were inhibited in both IKK α ^{-/-} and IKK β ^{-/-} MEFs. These results indicated that c-Jun, ATF2 and Fra-1 were the major AP-1 components that were under the downstream of IKKs and involved in the cellular arsenite response.

To further confirm the role of IKK α and IKK β in the arsenite-induced AP-1 transactivation, the reconstituted IKK α ^{-/-} (IKK α) (Fig. 2A) and IKK β ^{-/-} (IKK β) cells (Fig. 2D) were transiently transfected with the AP-1 luciferase reporter construct. We found that reintroduction of IKK α or IKK β into the respective null cells could rescue the arsenite-induced AP-1 transactivation (Fig. 2B and 2E). However, the peak induction of AP-1 activity under arsenite exposure in the reconstituted cells was later (24 and 48hrs in the reconstituted IKK β ^{-/-} and IKK α ^{-/-} cells, respectively) compared with that in the wild-type cells (12hrs, Fig. 2B and 2E), correlating with the delayed appearance of the apoptotic feature in the reconstituted cells (Fig. 1C and 1D). Further evidence showed that the alteration of c-Jun and ATF2 phosphorylation and the induction of Fra-1 can be restored in the arsenite-treated IKK α ^{-/-} (IKK α) and IKK β ^{-/-} (IKK β) cells (Fig. 2C and 2F), suggesting that the concomitant presence of both IKK α and IKK β was required for the arsenite-induced AP-1 transactivation in MEFs

JNKs and p38K pathways were the downstream cascades of IKK that mediated arsenite-induced AP-1 transactivation

JNKs and p38 kinase pathways are the most important mediators of AP-1 transactivation under various stress conditions [7,10,11]. To further address the functional link between IKKs and AP-1 activation in the arsenite response, we next compared the activation status of these two MAPKs in the wild-type, $IKK\alpha^{-/-}$ and $IKK\beta^{-/-}$ MEFs. As shown in Fig. 3A, 20 μ M of arsenite exposure induced the efficient activation of both JNKs and p38 kinase in the wild-type cells, which events were totally blocked by either $IKK\alpha$ or $IKK\beta$ deficiency. MKK7 is a MAPK kinase (MAPKK) that serves as the specific activator of JNKs pathway [24]; whereas MKK4 is the MPKK that responsible for the activation of both JNKs and p38K pathways [25]. MKK3 and MKK6 are two closely related dual-specificity MAPKKs which specifically targets on p38K activation [26]. We found that arsenite stimulation induced the significant phosphorylation of MKK4 and MKK3/6, while MKK7 is constitutively activated in the unstimulated wild-type cells and its activation status did not show obvious change after arsenite exposure. $IKK\alpha$ or $IKK\beta$ deficiency abolished arsenite-induced MKK4 and MKK3/6 phosphorylation, but did not affect MKK7 activation (Fig. 3A). These results indicated that MKK3/4/6 and JNKs/p38K might be the sequential downstream mediators of IKKs that were involved in the arsenite-induced AP-1 activation. More evidence from the reconstituted cells showed that re-introduction of $IKK\beta$ or $IKK\alpha$ into the null cells can efficiently restored both MKK3/4/6 and JNKs/p38K activation (Fig. 3B and 3C), further confirmed the roles of JNKs and p38K cascades in delivering IKKs-mediated arsenite response.

To provide direct evidence that both JNKs and p38K pathways activation are responsible for AP-1 induction under arsenite exposure, the plasmids expression DN-MKK4 and DN-MKK7 were co-transfected into the wild type cells and the stable transfectants were obtained (Fig. 3D). We observed that co-transfection of DN-MKK4 totally blocked JNKs activation and significantly attenuated p38K phosphorylation as well (Fig. 3E). Consistently, arsenite-induced AP-1 transactivation and its components phosphorylation/induction were blocked in the DN-MKK4transfected cells compared with the vector control-transfectants (Fig. 3E and 3F). These results strongly demonstrated that JNKs and p38K pathways are the mediators of AP-1 induction in the arsenite response.

GADD45 α acted as the upstream activator of JNKs/p38K pathways in response to the arsenite exposure to mediate AP-1 transactivation

Our previous report has demonstrated that GADD45 α accumulation can be rapidly induced under the arsenite stimulation and therefore mediate the cross-talk between $IKK\beta$ and JNKs-dependent pathway to trigger the cell apoptotic effect in the fibroblast [21]. To further disclose the functional link between IKKs and JNKs/p38K pathways-mediated AP-1 transactivation, we next studied the role of GADD45 α in the AP-1 induction under arsenite stimulation. As shown in Fig. 4A, we repeatedly observed the sustained induction of GADD45 α expression in the wild-type cells, which event was totally blocked in both $IKK\alpha^{-/-}$ and $IKK\beta^{-/-}$ cells. In addition, reconstitution of $IKK\alpha^{-/-}$ and $IKK\beta^{-/-}$ cells restored GADD45 α induction (Fig. 4B and 4C), further confirmed the role of GADD45 α in IKKs-mediated arsenite response. When GADD45 α induction was interfered by its specific siRNA transfection in the wild type cells, the activation of MKK3/4/6 and JNKs/p38K were totally blocked in the absence of GADD45 α accumulation (Fig. 4D). Under the same conditions, activation of c-Jun and ATF2 and the induction of Fra-1 were abolished (Fig. 4E), which were consistent with the significant reduction of AP-1 transactivation in GADD45 α siRNA-transfected wild-type cells compared with the control siRNA-transfectant (Fig. 4F). Taken together, we have identified IKKs/GADD45 α /MKKs/JNKs-p38K pathways for the mediation of the arsenite-induced AP-1 transactivation.

Arsenite-induced AP-1 transactivation was mediated by NF- κ B p50 subunit and unrelated to the classical NF- κ B transcriptional activity

As similar as AP-1, NF- κ B transactivation also can be induced by arsenite at various target cells and plays important role in the arsenite-induced cell life and death decision [16,17]. As IKKs has been proved to act as the upstream mediators of AP-1 induction, we wondered whether there was any relationship between the activation of these two important transcription factors, both of which were under the control of IKKs in the arsenite response.

We firstly detected the activation of NF- κ B in the wild-type, IKK $\alpha^{-/-}$ and IKK $\beta^{-/-}$ cells by gel shift assay using oligonucleotide containing consensus NF- κ B-binding sequence as a probe. As shown in Fig. 5A, arsenite stimulation induced a rapid and strong protein-DNA complex formation in the wild-type cells, which phenomena was absent in IKK α and IKK β -null cells at the same time points. This result indicated that arsenite-induced NF- κ B activation depended on the cooperative roles of IKK α and IKK β . The specificity of the gel shift assay was confirmed by the competition experiment, which showed that the protein-DNA complex formation in the wild-type cells was efficiently inhibited in the presence of 20-fold molar excess of the unlabeled NF- κ B cold probe (lane3 in Fig. 5B), but not of the same amount of the mutant cold probe (lane 5 in Fig. 5B). When super-shift assay was preformed by adding the anti-p65 or anti-p50 antibodies in the reaction mixture, we observed the shift of the major retarded band in the presence of the anti-p65 antibody, but not of the anti-p50 antibody (lane 6 and 7 in Fig. 5B), indicated that functional NF- κ B induced by arsenite stimulation was consisted of p65 but not of p50 subunit. This result was further confirmed in the p50 $^{-/-}$ MEFs (Fig. 5C), in which the expression of p65 subunit is intact (data not shown) and therefore NF- κ B activation induced by arsenite stimulation was as comparable as that in the wild-type cells (lane 2 and 4 in Fig. 5C) and the major DNA-protein complex can be identified by anti-p65 antibody (lane 5 in Fig. 5C). Super gel-shift assay in the nickel compound (NiCl₂)-treated human bronchial epithelial Beas-2B cells showed that transactivation of NF- κ B upon nickel exposure was delivered by both p65 and p50 subunits, which can be served as a positive control of the activity of p50 antibody in the above EMSA experiments (Fig. 5D).

Our previous data have demonstrated that GADD45 α induction in response to the arsenite stimulation was due to the inhibition of GADD45 α protein degradation but not of the transcription of the *GADD45 α* gene. In addition, this effect was mediated by p50 instead of p65 subunit [21]. Therefore, in combination of the above results from the gel shift assay, we proposed that GADD45 α -mediated downstream signaling events including the AP-1 transactivation seems have no relationship with NF- κ B activity, although they shared the common upstream mediators, IKK α and IKK β . To test this hypothesis, we compared the induction of AP-1 components in the p65 $^{-/-}$ and p50 $^{-/-}$ MEFs under the arsenite exposure. The results showed that arsenite-induced c-Jun and ATF2 phosphorylation and Fra-1 induction were significantly inhibited by p50 deficiency (Fig. 6A), but not affected, or even upregulated in the absence of p65 expression (Fig. 6B). Consistently, AP-1 transactivation was totally blocked in the p50 $^{-/-}$ MEFs (Fig. 6C), but showed comparable level between p65 $^{+/+}$ and p65 $^{-/-}$ MEFs under the same concentration of arsenite stimulations (Fig. 6D). Furthermore, reconstitution of p50 in the null cells restored arsenite-induced AP-1 activity (Fig. 6E). These results together indicated that IKKs-dependent AP-1 transactivation was mediated by p50 subunit of NF- κ B, but was unrelated with the classical NF- κ B transcriptional activity in the arsenite response.

AP-1 functions as an important effector for executing the cell death dictation from the IKKs signalsome in the arsenite response

We have demonstrated above that c-Jun, ATF2 and Fra-1 were the major AP-1 components in the arsenite response. Usually, Jun proteins can homodimerize or heterodimerize with ATF or

Fos family proteins; ATF proteins, on the other hand, form homodimers as well as heterodimers with Jun proteins. Fos proteins do not form stable homodimers but can bind DNA by forming heterodimers with Jun proteins that are more stable than Jun:Jun dimers [1,2]. Therefore, we proposed that the functional AP-1 induced by arsenite stimulation might be consist of the c-Jun:c-Jun, ATF2:ATF2 homodimers or the c-Jun: Fra-1, c-Jun:ATF2 heterodimers. Because it has been well-accepted that the c-Jun possesses the highest transcriptional activity among the multiple AP-1 proteins [1,2] and it was the major component of the various AP-1 dimers in the arsenite response, we thus tested if functional suppression of c-Jun induction by a dominant negative c-Jun mutant (TAM67) could affect the AP-1 activity and the cellular death response under the arsenite stress. Ectopic expression of TAM67 in WT cells was confirmed by the western-blotting assay [19]. As expected, TAM67 overexpression significantly inhibited the arsenite-induced c-Jun phosphorylation (Fig. 7A) and the AP-1 transactivation (Fig. 7B) compared with the vector control-transfected WT cells. Most importantly, the arsenite-induced cell death incidence was also attenuated by TAM67 transfection under the same conditions (Fig. 7C). These data indicated that c-Jun/AP-1 functions as a key executer under the downstream of the IKKs signalsome for eliciting the cell death effect in the arsenite response.

DISCUSSION

A critical role of the nuclear transcription factor AP-1 in the induction of the genetic programs regulating cell proliferation and apoptosis has been proved extensively. Therefore, AP-1 proteins are considered to be both oncogenic and anti-oncogenic [1–6]. Arsenite has been shown to exert either tumor-promoter or tumor-suppressor activity under the different conditions [16,17]. The efficient AP-1 transactivation under the various concentrations of arsenite stimulation has been observed in a plenty of target cells, which can mediate different, even opposing biological function. Persistent AP-1 activation has been implicated in the proliferation-enhancement effect of low dose of arsenic on the uroepithelial cells and keratinocyte, therefore contributes to the ability of arsenic to cause cancer [18,20]. However, high dose of arsenic induces AP-1 transactivation in the epidermal cells and keratinocyte, which is one of the mechanisms for the cytotoxicity of arsenic [6,19]. Given the importance of AP-1 activation in mediating the dual-role of arsenic, the molecular events leading to AP-1 induction under different arsenic exposure conditions are attractive.

We showed in this study that high dose of arsenite-induced AP-1 transactivation contributed to the cell apoptotic effect in mouse fibroblast, which event was mediated by both IKK α and IKK β -dependent and NF- κ B transcriptional activity-independent manner. This result provided a novel model of the cross-talk between the NF- κ B and AP-1 transcription pathways under the stress conditions. Previous studies have demonstrated that AP-1 functions as the subordinate signaling molecule under the downstream of NF- κ B, which activity depends on p65 subunit and is responsible for the regulation of the transcriptional induction of AP-1 components [14, 15]. By contrast, AP-1 transactivation was mediated by IKK/p50, but not of IKK/p65 pathway, under the arsenite stimulation; while the transcriptional activity of NF- κ B still depended on the presence of p65, but not of p50 subunit in the arsenite response (Fig. 5). Therefore, although shared with the common upstream protein kinases (IKKs), NF- κ B and AP-1 is believed to represent two “parallel” signaling pathways induced by arsenite through distinct mechanism (Fig. 8). Moreover, our previous data have shown that p65^{-/-} MEFs are more sensitive, while p50^{-/-} MEFs are resistant to the arsenite-induced cell apoptosis compared with the wild type control cells [21]. These data suggested that IKK/p65 seemed mediate cell survival, while IKK/p50 delivered cell apoptotic response under the same arsenite stimulation conditions. And the balance of these two pathways determined the final outcome of the arsenite effect.

Although both IKK α and IKK β were involved in the arsenite-induced AP-1 transactivation, their roles seemed distinct. Firstly, as shown in Fig. 2, reconstitution of IKK α and IKK β -null

cells restored arsenite-induced AP-1 transactivation and its components induction/phosphorylation with the different time course response. The alteration of the AP-1 components and their upstream signaling molecules under arsenite stimulation was observed in IKK $\beta^{-/-}$ (IKK β) cells as early as 12hrs after exposure compared with the occurrence of the same phenomena in IKK $\alpha^{-/-}$ (IKK α) cells 48hrs after stimulation, suggested that IKK β and IKK α might be involved in the AP-1 transactivation at early and late stage, respectively. However, we can only detect the persistent induction of the AP-1 components in the wild type cells within 24hrs of the arsenite exposure (data not shown), so how IKK α exert its function with so slow a response is unclear. Secondly, our previous data have demonstrated that the functional link between IKK β and IKK α with p50 was quite different [21]. Arsenite is able to induce a transient interaction of p50 specifically with IKK β , but not with IKK α , in the wild type MEFs, indicating that these two IKKs coupled to p50 and the subsequent signaling events including AP-1 activation with different mechanism. Thirdly, phosphorylation of IKK β , the prerequisite step for the activation of IKKs-dependent pathway, was totally blocked in the IKK $\alpha^{-/-}$ cells (Data not shown), suggested that IKK α might act as the upstream protein kinase for the mediation of IKK β activation and then these two IKKs cooperatively triggered the AP-1 transactivation and the cell death response, which is an interesting topic currently under investigation.

Accumulated evidence has demonstrated that two events are important for the regulation of AP-1 activity. The first is the phosphorylation of AP-1 components by the three kinds of MAPKs (JNKs, p38K and ERKs) and the second is the selective formation of the AP-1 dimers under certain conditions [1–6]. Our early study has demonstrated that JNKs play a key role in the arsenite-induced cell death effect [19,21]; while ERKs activation induced by arsenite mainly contributes to the tumor promotion activity [27]. Further investigation disclosed that MKK/JNKs pathway can be activated by arsenite through IKKs-dependent manner and target to the mitochondrial apoptosis-related proteins (Cytochrome C, Bid, Bcl-2) for triggering the cell death response via the intrinsic apoptotic pathway [21]. Moreover, data in this study additionally disclosed that MKK/JNKs cascade activation also mediated cell apoptosis through AP-1-dependent manner. Although we have not yet identified the downstream target gene(s) of AP-1, the results of the current study and the previously published data together strongly indicated that JNKs mediated arsenite-induced cell apoptosis via multiple mechanisms. p38K is another important mediator of the AP-1 transactivation under various stress conditions [7,10,11]. Data in this study have proved that MKK/p38K pathway was also involved in the AP-1 transactivation under the arsenite exposure cooperatively with JNKs. And this was the first report disclosing the cross-talk between IKKs and p38K pathways for the mediation of the cell death effect. We also observed the activation of MEK/ERKs pathway under the high dose of the arsenite stimulation (Data not shown). However, we excluded the role of ERKs in the cell death-related AP-1 induction for the reason that ERKs specific inhibitor, PD98059, can not alter high dose of arsenite-induced AP-1 transactivation and cell death response. In addition, the ERKs phosphorylation was a late stage response under the arsenite exposure, which can not be detected until 24hrs after arsenite treatment (Data not shown); while the peak AP-1 activation appeared as early as 12hrs according to the kinetic analysis of AP-1 induction (Fig. 1B). Therefore, the function of ERKs activation induced by high dose of arsenite stimulation need to be further addressed.

Due to the multi-function of the AP-1 proteins, the selection of the different AP-1 dimers is considered as another mechanism for the modulation of AP-1 activity [1,2]. Data in this study proposed that the functional AP-1 involving in the cytotoxic effect of arsenite was consist of the c-Jun or ATF2 homodimers or c-Jun:ATF2, c-Jun:Fra-1 heterodimers. Therefore, c-Jun is the key member of the various AP-1 dimers in the arsenite response. The predominant role of c-Jun in the arsenite-induced AP-1 transactivation and cell apoptosis was further confirmed by the dominant negative c-Jun mutant (TAM67) transfection, under which conditions the AP-1

activity was almost totally blocked and cell death incidence was partially reduced (Fig. 7). Previous reports regarding the role of ATF2 major focus on their involvement in the process of cell transformation and differentiation [28,29]. Similarly, the activity of Fra-1 is also observed to be related to the malignant transformation [30]. The results in this study disclosed the novel pro-apoptotic potential of ATF2 and Fra-1 upon arsenite exposure, which provided the additional anti-oncogenic effects of these two AP-1 components under the stress conditions.

Overall, data in this study illustrated the mechanism of the contribution of AP-1 transactivation to the arsenite-induced cell apoptotic effect, which was mediated by the activation of IKKs/p50/GADD45 α /MKKs/MAPKs signaling pathway, but was unrelated to the classical NF- κ B transcriptional activity. And the discovery of the anti-oncogenic effect of the three AP-1 components might provide the new insights for the targeted anticancer treatment by arsenite.

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Figure 1. Both IKK α and IKK β were involved in the arsenite-induced AP-1 transactivation and the accompanying cell apoptotic effect

(A) Identification of the wild type (WT), $IKK\alpha^{-/-}$, $IKK\beta^{-/-}$ MEFs. (B) The WT, $IKK\alpha^{-/-}$ and $IKK\beta^{-/-}$ MEFs were transfected with the AP-1 luciferase reporter plasmid and the stable transfectants were treated with a single dose of arsenite (20 μ M) for the different time periods. AP-1 transactivation was detected and the results were presented as the relative AP-1 induction which normalized to the control cells without any treatment. (C–D): The WT, $IKK\alpha^{-/-}$, $IKK\beta^{-/-}$ and the reconstituted $IKK\alpha^{-/-}$ ($IKK\alpha$), $IKK\beta^{-/-}$ ($IKK\beta$) MEFs were treated with arsenite (20 μ M) for 48hrs and then cell death were observed under microscope (C) or determined by the trypan blue exclusion assay (D). (E): The WT, $IKK\alpha^{-/-}$ and $IKK\beta^{-/-}$ MEFs

were treated with different dose of arsenite for 12hrs and the activation or the induction of the different AP-1 components was detected.

Figure 2. Reconstitution of IKK α or IKK β into the individual null cells restored the arsenite-induced AP-1 transactivation

(A and D): Identification of the reconstituted IKK $\alpha^{-/-}$ (IKK α) and IKK $\beta^{-/-}$ (IKK β) MEFs. (B) The WT, IKK $\beta^{-/-}$ and the reconstituted IKK $\beta^{-/-}$ (IKK β) MEFs were transiently transfected with the AP-1 luciferase reporter plasmid. The cells were treated with arsenite (20 μ M) 48hrs after transfection and then the AP-1 transactivation were detected at the different timepoints indicated. (C) The WT, IKK $\beta^{-/-}$ and the reconstituted IKK $\beta^{-/-}$ (IKK β) MEFs were treated with arsenite for 12hrs and then the activation or induction of the AP-1 components were detected. (E) WT, IKK $\alpha^{-/-}$ and the reconstituted IKK $\alpha^{-/-}$ (IKK α) MEFs were transiently transfected with the AP-1 luciferase reporter plasmid. The cells were treated with arsenite

(20 μ M) 48hrs after transfection and then the AP-1 transactivation were detected at the different timepoints indicated. (F) WT, IKK $\alpha^{-/-}$ and the reconstituted IKK $\alpha^{-/-}$ (IKK α) MEFs were treated with arsenite and then the activation or induction of AP-1 components were detected 12hrs (left panels) or 48hrs (right panels) later.

Figure 3. JNKs and p38K signaling cascades were the activators of AP-1 under the downstream of IKKs in the arsenite response

(A): The WT, IKK α ^{-/-} and IKK β ^{-/-} MEFs were treated with the different doses of arsenite for 12hrs and the activation of MKK3/6/4/7, JNKs and p38K were detected. (B): The WT, IKK β ^{-/-} and the reconstituted IKK β ^{-/-} (IKK β) MEFs were treated with arsenite (20 μ M) for 12hrs and the same western-blot assay was performed as described in (A). (C): The WT, IKK α ^{-/-} and the reconstituted IKK α ^{-/-} (IKK α) MEFs were treated with arsenite (20 μ M) for 12 or 48hrs and the same western-blot assay was performed as described in (A). (D) The WT cells were co-transfected with the dominant negative mutant of MKK4 and MKK7 and the stably transfectant was identified. (E) WT/control vector or WT/DN-MKK4 stable

transfectants were treated with arsenite for 12hrs and the activation of JNKs, p38K pathways and the induction of the AP-1 components were detected. (F) WT/control vector or WT/DN-MKK4 stable transfectants were transiently transfected with the AP-1 luciferase reporter plasmid. The cells were then treated with arsenite 48hrs after transfection and AP-1 activity was detected 12hrs after exposure.

Figure 4. GADD45 α acted as the upstream activator of JNKs/p38K pathways in response to the arsenite exposure to mediate AP-1 transactivation

(A) The WT, IKK α ^{-/-} and IKK β ^{-/-} MEFs were treated with 20 μM of arsenite for the time indicated and then the expression of GADD45 α was detected. (B) The WT, IKK β ^{-/-} and the reconstituted IKK β ^{-/-} (IKK β) MEFs were treated with arsenite (20 μM) for 12hrs and then the induction of GADD45 α expression was detected (C) The WT, IKK α ^{-/-} and the reconstituted IKK α ^{-/-} (IKK α) MEFs were treated with arsenite (20 μM) for 12hrs and then the induction of GADD45 α expression were detected. (D–E) The WT cells were transfected with GADD45 α siRNAs mixture or the control siRNAs and then the according stable transfectants were treated with arsenite for 12hrs. The activation of the MKK/JNKs and MKK/p38K pathways (D) and

the activation or induction of the AP-1 proteins (E) was detected. (F) WT/control siRNAs or WT/GADD45 α siRNAs stable transfectants were transiently transfected with the AP-1 luciferase reporter plasmid. The cells were treated with arsenite 48hrs after transfection and the transactivation of AP-1 were detected 12hrs after exposure.

Figure 5. NF- κ B transactivation under arsenite exposure was mediated by p65 subunit
 (A) The WT, IKK $\alpha^{-/-}$ and IKK $\beta^{-/-}$ MEFs were treated with 20 μ M of arsenite for the time indicated and then the DNA binding ability of NF- κ B was detected by gel shift assay taking the oligonucleotide containing the consensus NF- κ B binding sequences as a probe. (B) A 5 or 20-fold molar excess of the unlabeled NF- κ B or the mutant NF- κ B cold probe was added to the binding reaction mixtures of the WT MEFs to determine the binding specificity of the EMSA experiment. Super-gel shift assay was performed in the presence of the antibody specific for p65, p50 or the pre-immune serum, respectively. (C) The WT and p50 $^{-/-}$ MEFs were treated with arsenite for 4hrs and the NF- κ B DNA binding ability was detected. The super-gel shift assay was also determined in the p50 $^{-/-}$ MEFs in the presence of anti-p65 antibody. (D)

Beas-2B cells were treated with NiCl_2 for 12hrs and then DNA binding ability of NF- κ B was detected. Super-gel shift assay was performed in the presence of the antibody specific for p65, p50 or the pre-immune serum, respectively.

Figure 6. AP-1 transactivation under arsenite exposure was delivered by NF-κB p50 instead of p65 subunit

(A and B) The p50^{-/-} or the p65^{-/-} MEFs and their corresponding wild type control cells were treated with arsenite for 12hrs and the induction of GADD45α and the AP-1 components were detected. (C and D) The p50^{-/-} or the p65^{-/-} MEFs and their corresponding wild type control cells were transiently transfected with the AP-1 luciferase reporter plasmid. The cells were then treated with arsenite 48hrs after transfection and the AP-1 activity was detected 12hrs after exposure. (E) The reconstituted p50^{-/-} (p50) MEFs were transiently transfected with the AP-1 luciferase reporter plasmid. The cells were then treated with arsenite 48hrs after transfection and the AP-1 activity was detected at the different timepoints indicated.

Figure 7. AP-1 was the mediator of the arsenite-induced cell apoptotic response

(A) The WT cells were transfected with the dominant negative mutant of c-Jun (TAM67) or the control vector and the stable transfectants were obtained. The cells were treated with arsenite for 12hrs and the activation of c-Jun was detected. (B) WT/control vector or WT/TAM67 stable transfectants were transiently transfected with the AP-1 luciferase reporter plasmid. The cells were treated with arsenite 48hrs after transfection and the transactivation of AP-1 were detected 12hrs after exposure. (C) WT/control vector or WT/TAM67 stable transfectants were treated with arsenite for 48hrs and then cell death were determined by the trypan blue exclusion assay.

Figure 8. Proposed scheme of the signaling cascades mediated arsenite-induced cell apoptotic response

Under the conditions of high dose of arsenite stimulation, $IKK\alpha$ and $IKK\beta$ cooperatively transduced the signal to the NF- κ B p50 subunit, which could increase the stability of GADD45 α and then triggered the activation of MKK/JNKs and MKK/p38K cascades. By inducing the mitochondrial dysfunction and AP-1 transactivation, IKKs mediated cell apoptotic effect through both intrinsic and extrinsic pathways. High dose of arsenite also induced NF- κ B transactivation, which was dependent on p65 but not of p50 subunit.